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# Pituitary Adenylate Cyclase-Activating Polypeptide and Islet Amyloid Polypeptide in Primary Sensory Neurons

## *Functional Implications from Plasticity in Expression on Nerve Injury and Inflammation*

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## Abstract

Primary sensory neurons serve a dual role as afferent neurons, conveying sensory information from the periphery to the central nervous system, and as efferent effectors mediating, e.g., neurogenic inflammation. Neuropeptides are crucial for both these mechanisms in primary sensory neurons. In afferent functions, they act as messengers and modulators in addition to a principal transmitter; by release from peripheral terminals, they induce an efferent response, "neurogenic inflammation," which comprises vasodilatation, plasma extravasation, and recruitment of immune cells. In this article, we introduce two novel members of the sensory neuropeptide family: pituitary adenylate cyclase-activating polypeptide (PACAP) and islet amyloid polypeptide (IAPP). Whereas PACAP, a vasoactive intestinal polypeptide-resembling peptide, predominantly occurs in neuronal elements, IAPP, which is structurally related to calcitonin gene-related peptide, is most widely known as a pancreatic β-cell peptide; as such, it has been recognized as a constituent of amyloid deposits in type 2 diabetes. In primary sensory neurons, under normal conditions, both peptides are predominantly expressed in small-sized nerve cell bodies, suggesting a role in nociception. On axotomy, the expression of PACAP is rapidly induced, whereas that of IAPP is reduced. Such a regulation of PACAP suggests that it serves a protective role during nerve injury, but that of IAPP may indicate that it is an excitatory messenger under normal conditions.

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In contrast, in localized adjuvant-induced inflammation, expression of both peptides is rapidly induced. For IAPP, studies in IAPP-deficient mice support the notion that IAPP is a pronociceptive peptide, because these mutant mice display a reduced nociceptive response when challenged with formalin.

**Index Entries:** PACAP; amylin; neuropeptides; IAPP; *in situ* hybridization; neurogenic inflammation; β-cells; axotomy; Freund's complete adjuvant; knockout mice.

## Introduction

### *Neuropeptides in Primary Sensory Neurons*

It has long been recognized that primary sensory neurons express a variety of peptide messengers, often termed neuropeptides (for a review, *see* Hökfelt, 1991). In particular, small-sized neurons, which issue C-type unmyelinated fibers and which are believed to be involved in pain and temperature transmission, are the ones most commonly associated with neuropeptide expression. Neuropeptides in primary sensory neurons are messengers in addition to a principal transmitter, e.g., glutamate is considered to be the excitatory transmitter in many primary sensory neurons (cf. Salt and Hill, 1983). At the subcellular level, neuropeptides in primary sensory neurons are believed to be contained within large dense core vesicles, whereas the principal transmitter is stored in small clear vesicles, although it may also occur in the former. This may have functional implications, because the large dense core vesicles are released in response to bursting or high-frequency activity, whereas the small vesicles are released by normal firing rates (for a review, *see* Hökfelt, 1991). These mechanisms correlate with the fact that the response to peptide release is often sustained, but that to the principal transmitter is rapid but brief.

It has also become clear that primary sensory neurons release neuropeptides both from their central terminals in the dorsal horn of the spinal cord and from free nerve endings in peripheral tissues (reviewed in Holzer, 1988). Thus, although stimulation of peripheral afferents conveying sensory impulses to the dorsal horns of the spinal cord may be the primary function of the primary sensory neurons, antidromic activation of these afferents causes the release of transmitter from the peripheral terminals of the activated neuron (for reviews, *see* Maggi and Meli, 1988; Holzer, 1988). It is believed that sensory afferents through this "axon reflex" exert a local effector function, which comprises vasodilatation and plasma extravasation as well as recruitment and activation of immune cells (for reviews, *see* Holzer, 1988; Szolcsanyi, 1988; Barnes et al., 1990). Moreover, it has become clear that the "classical" transmitters are not responsible for this interaction of immune and neuronal mechanisms, termed neurogenic inflammation (Holzer, 1988; Barnes et al., 1990). Instead, a crucial role for neuropeptides has been suggested by observations that pretreatment with capsaicin, an extract of pepper that depletes sensory nerves of peptides, abolishes these responses (Holzer, 1988).

Under normal conditions, the most ubiquitously expressed neuropeptides are calcitonin gene-related peptide (CGRP), which occurs in approximately half of all sensory neurons (Noguchi et al., 1993; Mulder et al., 1994), and substance P (SP), which is found in 20% of sensory neurons (Noguchi et al., 1988, 1989). CGRP occurs as two highly homologous variants (α- and β-CGRP), which are encoded by two distinct genes (Amara et al., 1985); in primary sensory neurons, α-CGRP predominates, but β-CGRP is also found (Mulderry et al., 1988). The preprotachykinin (PPT) gene A, which encodes SP, also gives rise to precursors

from which neurokinin A and neuropeptide K can be formed (Kawaguchi et al., 1986; Krause et al., 1987); these peptides together with SP are designated as tachykinins and are also expressed in predominantly small-sized nerve cell bodies (Sundler et al., 1985). Also, somatostatin can be found in a population of small-sized nerve cell bodies (Noguchi et al., 1993; Zhang et al., 1993a). Cholecystokinin (CCK), on the other hand, is only rarely encountered in rat primary sensory neurons under normal conditions, but is constitutively expressed in, e.g., monkey dorsal root ganglion (DRG) neurons (Verge et al., 1993). Furthermore, vasoactive intestinal polypeptide (VIP) (Noguchi et al., 1989) and galanin (Villar et al., 1989; Zhang et al., 1993a) are expressed in minor populations of small-sized nerve cell bodies. The function and expression, however, of neuropeptides have also been studied under experimental and pathological conditions. It has become evident that neuropeptides may play an important role under such circumstances, of which peripheral axotomy and inflammation have been most extensively studied.

## Peripheral Axotomy

The study of peripheral nerve injury is of great clinical relevance, because it is frequently encountered in patients after traumatic injuries. As a result, patients may lose both motor and sensory functions to varying degrees, leading to debilitation. It represents a great challenge to limit the consequences of nerve injury and, possibly, to restore nerve function. Therefore, not only the acute perturbation and adaptation of nerve function after nerve injury, but also the long-term alterations, which may affect nerve regeneration, are important to study.

In rodents, a number of experimental models have been developed to enable study of these phenomena. Although many nerves are candidates for experimental injury, the sciatic nerve is most commonly used; the nerve is exposed at the sciatic notch and sectioned. To prevent regeneration, the proximal part of the distal nerve is removed. If regeneration is to be studied, the nerve can be repaired with epineurial sutures in a fashion similar to what is done in the clinical situation (Urabe et al., 1995). An alternative regenerative model is the nerve crush model, where axotomy is induced with a pair of jeweller's forceps or a ligature tied around a glass rod (cf. Danielsen et al., 1986).

After nerve injury, it is reasonable to assume that the activity of the neuron adapts to the injury situation, in order to limit its consequences and promote survival and regeneration of the neuron (cf. Lieberman, 1971). Consequently, activities of the neuron related to chemical transmission are downregulated, whereas its synthetic machinery is geared toward factors that facilitate regeneration (Grafstein and Forman, 1980). Axotomy may also lead to the loss of DRG neurons with degeneration of their central projections in the dorsal horn, a process termed transganglionic degeneration (for a review, *see* Aldskogius et al., 1985).

Also, the neuropeptide phenotype of primary sensory neurons changes ("messenger plasticity") on axotomy, conceivably to facilitate the adaptation of the neuron to the injury. Not surprisingly, expression of SP and CGRP, known to be excitatory neuropeptides, is downregulated (Jessell et al., 1979; Henken et al., 1990; Noguchi et al., 1990, 1993), thus befitting a situation in which signaling is not a primary concern of the neuron. Accordingly, expression of neuropeptide Y (NPY) and galanin, but also that of VIP, is either induced or markedly increased (Villar et al., 1989; Doughty et al., 1991; Noguchi et al., 1993; Zhang et al., 1993a); NPY and galanin are known as inhibitory neuropeptides. Thus, the plasticity of neuropeptide expression largely fits with the concept of sensory neuropeptides having, in addition to neuromodulation, other functions, such as promotion of growth and enhancement of neuronal survival (Schwartz, 1992; Pincus et al., 1994); these properties would be beneficial in an injured state and

may also serve antinociceptive purposes (Hökfelt et al., 1994).

### Adjuvant-Induced Inflammation

As described above, neuropeptides are crucially involved in neurogenic inflammation, which is believed to be an important component of peripheral inflammation. Such inflammation is an appropriate response in many situations, for instance, to combat the invasion of infectious agents. However, in several diseases, inflammation may also become inappropriate, perhaps triggered and perpetuated by autoimmune mechanisms, and may manifest itself as the predominant symptom. A large group of such diseases, e.g., rheumatoid arthritis, affects many patients all over the world.

A number of experimental models have also been developed for the study of these mechanisms. A well-established agent in this context is Freund's complete adjuvant (FCA), which induces arthritic inflammation when administered to rodents (Millan et al., 1988; Donaldson et al., 1993). The basis for this reaction is believed to be a crossreaction of antibodies to the mycobacterial antigens in FCA with that of antigens in synovial membranes. When administered subcutaneously at higher doses, FCA will induce systemic arthritis, whereas a lower subcutaneous dose, injected locally over a joint, will produce inflammation, which evolves into a mild localized arthritis (Millan et al., 1988; Donaldson et al., 1993). The advantage of this approach is that when studying responses in the innervating sensory neurons, alterations owing to systemic inflammatory effects are less likely to contribute. Moreover, paired comparisons are applicable, using the contralateral neurons as control.

The most important mediators of neurogenic inflammation are believed to be the tachykinins, SP in particular, and CGRP. These neuropeptides are localized to sensory afferents involved in inflammation (Uddman et al., 1986; Weihe et al., 1988) and are released on inflammation (Helme et al., 1986). Moreover, when administered to animals, they induce the signs that are typical of neurogenic inflammation (Brain and Williams, 1988). In mice with a targeted disruption of the PPT A gene, resulting in lack of SP and neurokinin A, neurogenic inflammation is virtually lost (Cao et al., 1998). Thus, compelling evidence for the importance of neuropeptides in inflammation exists. This also indicates the potential of neuropeptide antagonists in treatment of inflammatory diseases. Thus far, however, clinically useful compounds have not emerged.

Localized inflammation also alters the expression of neuropeptides in innervating sensory neurons; it has been found that expression of neuropeptides involved in neurogenic inflammation is upregulated. Thus, the level of PPT mRNA is increased in the L5 DRG as early as 3 h after a noxious stimulus (Noguchi et al., 1988) and after unilateral adjuvant-induced inflammation, levels of PPT and CGRP mRNA are rapidly increased in innervating rat DRG nerve cell bodies (Donaldson et al., 1992; Garrett et al., 1995). Similarly, the content of SP and CGRP in the ipsilateral sciatic nerve, DRG, and dorsal horn (Donnerer et al., 1992), and the number of CGRP-containing DRG neurons (Hanesch et al., 1993) increase after unilateral adjuvant-induced inflammation. Moreover, adjuvant-induced arthritis is associated with intensified SP/CGRP immunostaining, and sprouting of such fibers is found in the inflamed tissue (Weihe et al., 1988).

## Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

### Background

PACAP is a member of a superfamily of structurally related peptides that includes secretin, glucagon, glucagon-like peptide 1, VIP, and growth hormone-releasing factor. The peptide was first isolated from the ovine hypothalamus (Miyata et al., 1989), and its name is based on its ability to stimulate adenylate cyclase in cultures of rat anterior pituitary cells. It occurs in two biologically active forms:

PACAP-38 is the predominant form in most tissues, but PACAP-27 is a C-terminally truncated peptide; both are amidated at the C-terminus (Miyata et al., 1990). Subsequently, cloning has revealed that the amino acid sequence for PACAP-38 is identical in sheep, rats, and humans (Miyata et al., 1990; Ogi et al., 1990). At present, three receptors have been identified that are activated by PACAP: the $PAC_1$, $VPAC_1$, and $VPAC_2$ receptors (Lutz et al., 1995; Harmar et al., 1998). Two of these, $VPAC_1$ and $VPAC_2$, are also high-affinity receptors for VIP. The $PAC_1$ receptor occurs in several splice variants, whereas splice variants of the other two receptors have not been described to date.

PACAP is expressed in a variety of tissues, such as brain, the hypothalamus in particular, adrenal gland, and testis (Ghatei et al., 1993; Shioda et al., 1994; Hannibal and Fahren Krug, 1995; Frodin et al., 1995; Steenstrup et al., 1995; Gras et al., 1996). Moreover, PACAP immunoreactivity is found in neuronal elements in the airways (Cardell et al., 1991; Uddman et al., 1991), gastrointestinal tract (Shen et al., 1992; Sundler et al., 1992; Köves et al., 1993; Hannibal et al., 1998), pancreas (Fridolf et al., 1992), female genital tract (Steenstrup et al., 1995), salivary gland (Tobin et al., 1994), and ocular tissues (Wang et al., 1995).

## PACAP in Primary Sensory Neurons

In 1993, the first indication that PACAP is a sensory neuropeptide was put forth (Moller et al., 1993). In this study, we demonstrated, using immunocytochemistry, that PACAP-immunoreactive nerve fibers are present in the superficial layers of the dorsal horn of the spinal cord at the cervical, thoracic, and lumbar levels, displaying a distribution similar to that of CGRP and SP (for a review, *see* Sundler et al., 1985). In fact, immunoreactive PACAP occurred in a subpopulation of CGRP-containing fibers. The DRG and trigeminal ganglion contained PACAP-immunoreactive nerve cell bodies of mostly small diameter; these neurons also expressed CGRP and SP. Colchicine pretreatment increased the number and the staining intensity of the PACAP-immunoreactive neurons. Accordingly, radioimmunoassay (RIA) demonstrated a relatively high concentration of PACAP in the spinal cord, although lower than that of CGRP (Mulderry et al., 1988); capsaicin treatment reduced the spinal levels of both peptides (Moller et al., 1993).

The next line of evidence establishing PACAP as a sensory neuropeptide was *in situ* hybridization, which demonstrated that PACAP mRNA indeed occurs in nerve cell bodies in the DRG (Fig. 1) and trigeminal ganglion (Mulder et al., 1994). Here, the vast majority of the PACAP mRNA-expressing neurons were of small to medium size. CGRP mRNA was expressed in a greater number of nerve cell bodies than PACAP mRNA; an estimation of the number of PACAP mRNA-containing nerve cell bodies in the DRG showed that PACAP was expressed in ≈10% of the total population of such neurons, when ganglia from the lumbar, thoracic, and cervical levels were included. CGRP, on the other hand, occurred in ≈46% of the total neurons. It was later found that the expression of PACAP in the spinal cord has an early onset, because in the rat, PACAP-containing fibers occur here as early as at embryonic day 13 (Nielsen et al., 1998). Moreover, in the mouse, using *in situ* hybridization, PACAP expression was demonstrated in neuronal structures as early as at embryonic day 9.5 (Sheward et al., 1998). The expression of PACAP in human primary sensory neurons has also been confirmed (Dun et al., 1996).

## Expression of PACAP After Axotomy

To determine whether expression of PACAP in primary sensory neurons is subject to change under pathological conditions, its expression in the L5 DRG on sciatic axotomy was examined (Zhang Q. et al., 1995). It was found that during normal conditions, 17.5% of all L5 DRG neurons expressed PACAP, and these were mainly of small size. Axotomy induced a rapid and prominent increase in

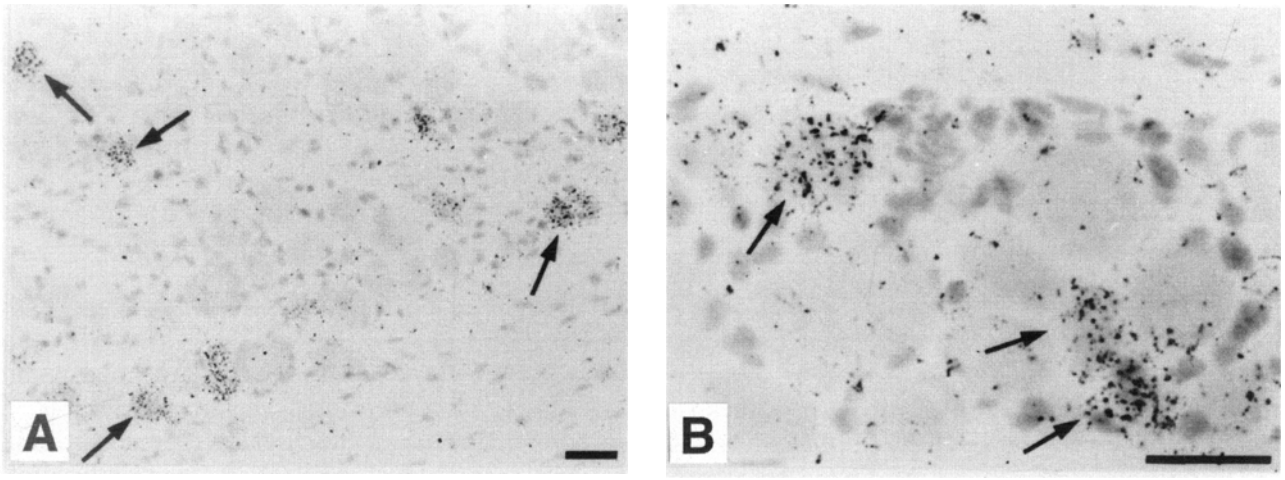


Fig. 1. *In situ* hybridization to PACAP mRNA in rat DRG. The localization of autoradiographic grains indicates that PACAP mRNA is predominantly expressed in a population of small-sized nerve cell bodies (arrows). Reproduced by kind permission from Elsevier Science. Bar = 10 μm.

PACAP expression; already at 15 h after transection, 35% of the neurons displayed PACAP immunoreactivity. At 3, 10, and 30 d after nerve injury, 51, 77, and 57%, respectively, of the DRG neurons were PACAP immunoreactive. This study indicated that also neurons of medium and large size express PACAP mRNA after axotomy. The intensity of immunocytochemical staining for PACAP immunoreactivity peaked at 3 d after axotomy. In addition, PACAP immunoreactivity accumulated proximal to a ligature applied around the sciatic nerve, indicating axonal transport of the peptide.

In a parallel axotomy study, using similar methods, but with the addition of immunochemistry and Northern blot analysis (Zhang Y. Z. et al., 1996), we found that the percentage of neurons expressing PACAP mRNA was 24% of the total number of DRG neurons in a section. At 14 d after sciatic axotomy, the number of L5 DRG neurons on the ipsilateral side expressing PACAP mRNA increased to 47% (Fig. 2). There was also a significant shift of PACAP mRNA expression from small-sized neurons on the uninjured side to medium- and large-sized neurons on the injured side (Fig. 3). Moreover, in the dorsal horn on the control side, PACAP-immunoreactive nerve fibers were densely accumulated in the superficial layers; after axotomy, this density was reduced in the medial part of the dorsal horn. The immunochemical analysis revealed that there was an increased concentration of PACAP-38 and PACAP-27 in the sciatic nerve after axotomy. An increased content of PACAP-38 in the DRG from level L5 and L6, but not L4, on the side of axotomy was also evident. In the spinal cord, the concentration of PACAP-27 was increased on the ipsilateral side, whereas a similar but lesser increase for PACAP-38 did not reach statistical significance. Moreover, Northern blot analysis demonstrated the occurrence of three different mRNA species (1.2, 2.4, and 3.0 kb) in lumbar DRG on the transected side; of these, the 2.4-kb species was most highly expressed, indicating a marked increase of PACAP mRNA. In the control DRG, only two species of PACAP mRNA were detected (2.4 and 3.0 kb).

Thus, both studies indicate that PACAP expression and PACAP content in sensory neurons are increased after sciatic axotomy. A similar upregulation of PACAP expression in

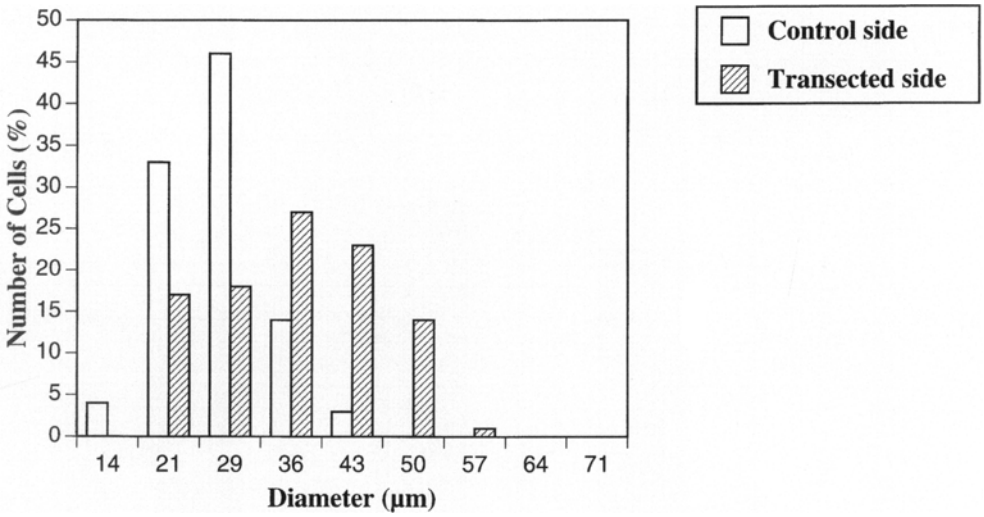


Fig. 2. Graphic representation of numbers of nerve cell bodies expressing PACAP mRNA at 14 d after axotomy. Data from Zhang Y. Z. et al. (1996).

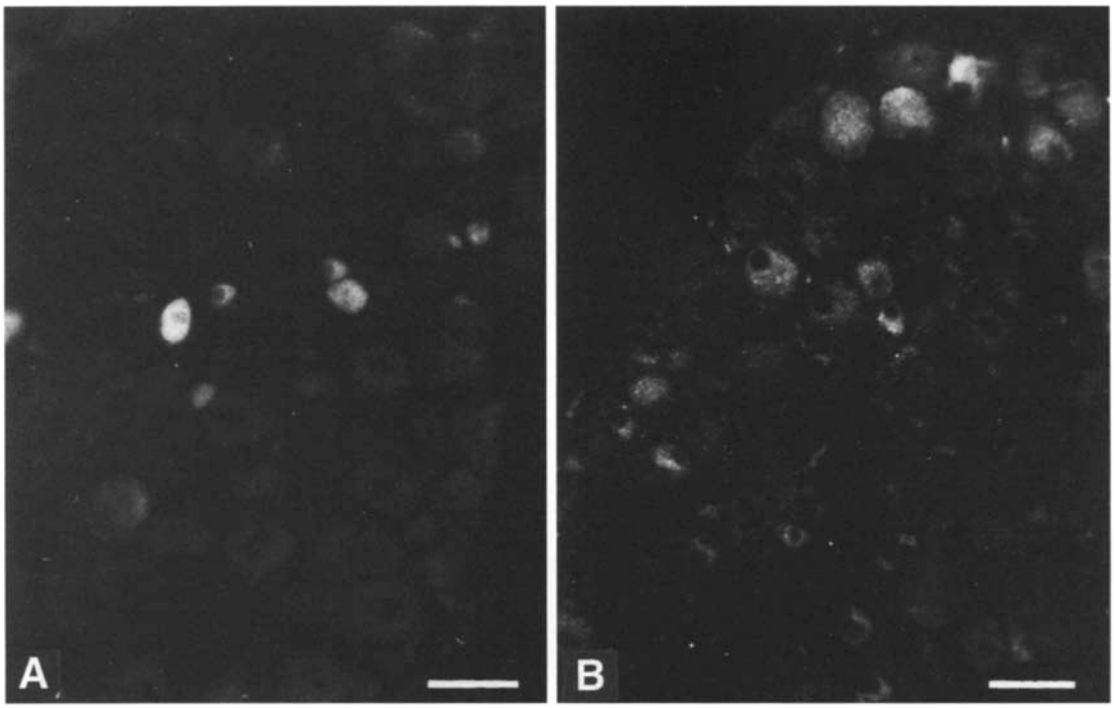


Fig. 3. Immunofluorescence for PACAP in L5 DRG contra- **(A)** and ipsilateral **(B)** to sciatic axotomy. On the control side (A), PACAP is expressed in a population of small-sized nerve cell bodies; on the side of axotomy at 14 d, the number of PACAP-expressing nerve cell bodies has increased and PACAP now also occurs in nerve cell bodies of large size. Reproduced by kind permission from Elsevier Science. Bar = 50 µm.

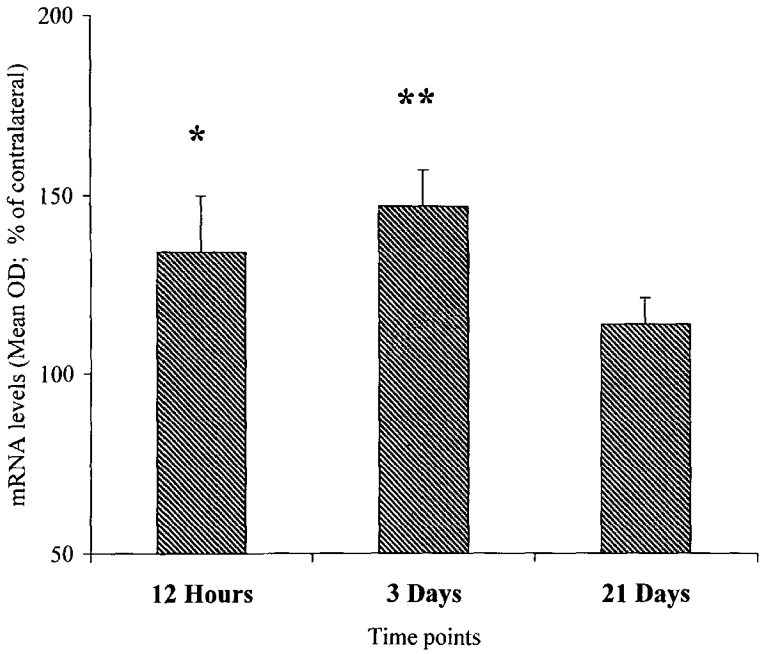


Fig. 4. PACAP mRNA levels in L5 DRG on adjuvant-induced inflammation as determined by quantitative *in situ* hybridization; mean optical density of labeling expressed as percent of that in the contralateral control DRG. Statistical comparisons at each time-point were made with a two-tailed paired Student's *t*-test; $*p < 0.05$, $**p < 0.01$. Data from Zhang et al. (1998); reproduced by kind permission from Rapid Science Ltd.

autonomic ganglia after nerve injury has also been described (Mulder et al., 1995b; Moller et al., 1997a,b). PACAP and its regulation in autonomic ganglia will, however, not be discussed further here; the readers are referred to the articles above and a recent review (Sundler et al., 1996).

## Expression of PACAP on Adjuvant-Induced Inflammation

As described above, the expression of neuropeptides implicated in neurogenic inflammation is upregulated in experimental models of inflammation. However, it was not known whether peripheral inflammation would affect PACAP expression. Therefore, we studied the expression of PACAP in innervating DRG in rats, in which a localized inflammation in the tarso-tibial region was induced by injection of FCA (Donaldson et al., 1993). As early as at 12 h after FCA injections, the number of PACAP mRNA-expressing neurons in the L5 DRG on the ipsilateral side was increased (Zhang et al., 1998). Furthermore, on the side of inflammation, the level of PACAP mRNA, as measured by mean optical density of *in situ* hybridization labeling in the DRG neurons, was increased (Fig. 4). This dual increase was also observed at 3 d after induction of inflammation, but it did not persist at 21 d after induction of inflammation.

The upregulation of PACAP expression after adjuvant-induced inflammation was an unexpected finding, because the peptide is also upregulated on axotomy. As discussed above, excitatory neuropeptides, e.g., CGRP, are upregulated after inflammation and downregulated on nerve injury, whereas inhibitory neuropeptides, such as galanin, display the opposite pattern: downregulation after inflammation and upregulation on axotomy (Hökfelt

et al., 1994). PACAP does not adhere to this pattern, because it is upregulated in both experimental situations (Zhang et al., 1995a, 1996a, 1998).

### *Functional Implications of PACAP in Primary Sensory Neurons*

Although PACAP was first isolated as a hypophysiotropic hormone, its role in the regulation of pituitary hormone secretion is still unclear (reviewed in Rawlings and Hezareh, 1996). It has been proposed that PACAP released from retinal afferents to the suprachiasmatic nucleus functions as a daytime regulator of the biological clock (Hannibal et al., 1995b). In the periphery, PACAP has been shown to regulate exocrine and endocrine pancreatic secretion (Filipsson et al., 1997, 1998; Yada et al., 1997) and to stimulate catecholamine secretion from the adrenal medulla (Przywara et al., 1996).

The role of PACAP in primary sensory neurons during physiological conditions is probably related to nociceptive transmission and modulation. The structural basis for this assumption is that nociceptive afferents, including C-type unmyelinated fibers, terminate in the superficial layers of the dorsal horn. In the sensory ganglia, PACAP is mainly found in small-sized nerve cell bodies (Moller et al., 1993; Mulder et al., 1994), inferred to issue Aδ- and C-fibers, known to be associated with polymodal and mechanical nociceptors. Additionally, most PACAP-containing fibers are present in laminae I and II of the dorsal horn (Moller et al., 1993; Zhang Y. Z. et al., 1996). Thus, in an early study, we found that PACAP-27 or PACAP-38 given intrathecally to rats elicits a long-lasting depression of a C-fiber-evoked flexion reflex (Zhang Y. Z. et al., 1993). The suggested antinociceptive role for PACAP has gained further supported from two independent studies, where intrathecally administered PACAP reduced formalin-induced pain behavior (Yamamoto and Tatsuno, 1995; Zhang et al., 1996b). However, all three studies used rather high concentrations of PACAP, raising the possibility that the effects observed may reflect pharmacological rather than physiological actions of the peptide. The proposed antinociceptive role of PACAP has recently been challenged by two other studies, in which PACAP was also administered intrathecally; in a rat model, lower concentrations of PACAP-27 than used previously induced a concentration-dependent facilitation of the flexor reflex (Xu and Wiesenfeld-Hallin, 1996), and in mice, PACAP produced a concentration-dependent decrease of the tail-flick latency (Narita et al., 1996). These two studies thus suggest that PACAP plays a nociceptive, rather than an antinociceptive role, and these ambiguities have not yet been resolved. There are, however, several possible explanations for these differences, such as the use of different concentrations of the peptide, model-specific discrepancies, and species differences. In yet another study, the physiological role for PACAP and VIP was examined using extracellularly recorded electrophysiological activity of single multireceptive dorsal horn neurons and ionophoretic administration of the two peptides (Dickinson et al., 1997); this study provides for the first time direct evidence that PACAP is an effective excitant of spinal dorsal horn neurons and also that PACAP is more potent than VIP in terms of neuronal excitation. The authors suggest that PACAP induces sensitization at the spinal cord level and that the source of endogenous PACAP is likely to include afferent C-fibers. Another difference between the two peptides is that VIP appears to be highly selective for multireceptive neurons only, whereas PACAP also excited a subpopulation of nonnociceptive neurons. The authors conclude that the previous morphological findings of PACAP-containing fibers in lamina I and II in the dorsal horn and additional fibers in lamina VII and around the central canal, which suggested a role for PACAP in control of normal sensory transmission, now has gained neurophysiological support (Dickinson et al., 1997).

On a complete nerve transection, the normal sensory input from the periphery is totally lost, at least in the acute phase, and during the regenerative phase, it is altered. The rapid increase of PACAP content and expression and also recruitment of larger-diameter neurons after nerve injury (Zhang et al., 1995a, 1996a) may reflect an altered or absent afferent input to primary sensory neurons. The functional implications of this increased PACAP synthesis after axotomy are unclear. It may, however, alter the sensory transmission conveyed in larger fibers, because an increased PACAP immunoreactivity has been demonstrated in the nucleus gracilis on the ipsilateral side after sciatic nerve transection (Jongsma et al., 1998); the nucleus gracilis is known to receive myelinated fibers emanating from large-sized nerve cell bodies in the DRG. Increased PACAP expression is not likely to reflect acute pain after nerve injury, because increased PACAP production occurs predominantly in nerve cell bodies of large diameter (Zhang et al., 1995a, 1996a), known to be involved in the modality of touch. Furthermore, it is clinically well known that patients experience no acute pain from the nerve transection itself, besides pain elicited from the wound. The pre-eminent problem in this situation is the loss of sensation, and if pain occurs, it is usually a late phenomenon related to regeneration and eventual neuroma formation (Mackinnon and Dellon, 1988).

The very rapid upregulation of PACAP under all the experimental conditions examined so far suggests that PACAP may have another role in primary sensory neurons than that commonly associated with neuropeptides. One possibility is that PACAP expression may be increased to serve a neuroprotective/neuroregenerative role (Arimura et al., 1994), and this has recently received some experimental support, because PACAP has been shown to protect DRG neurons from death in vitro (Lioudyno et al., 1998). Thus, PACAP has been shown to stimulate survival, growth, neurite formation and differentiation of several neuronal cell types in vitro and, in some instances, also in vivo; for example, PACAP prevents ischemia-induced death of hippocampal neurons (Uchida et al., 1996). In the mouse embryo, PACAP is an early inducer of cAMP3 levels, and may act in the neural tube during patterning to control cell proliferation and gene expression (Waschek et al., 1998). Along these lines, PACAP stimulates cell survival in rat cerebellar granule neurons through activation of protein kinase A, but also induces c-*fos* (Vaudry et al., 1998); this suggests that two pathways are responsible for both the short- and long-term effect of PACAP on cell survival. Additionally, in cultured rat astrocytes (Moroo et al., 1998) and cerebellar granule neurons (Villalba et al., 1997), PACAP stimulates mitogen-activated protein kinase, which is known to be involved in cell replication. Thus, evidence is accumulating that PACAP may be involved in neuroprotection/neuroregeneration, and these reports support the notion that increased PACAP expression on nerve injury may have implications for the injury itself and the process of nerve regeneration (*see also* Sundler et al., 1996). However, this putative neuroprotective effect of PACAP may be dependent on a number of factors yet to be clearly identified. For instance, although we found a similar upregulation of PACAP in cultured rat vagus nerves (Reimer et al., 1998) as in rat DRG (reviewed in Sundler et al., 1996), direct administration of the peptide to cultured rat vagus nerves did not affect the measures of regeneration of these nerve fibers (Reimer et al., 1998).

The upregulation of PACAP expression in sensory neurons in response to induced inflammation may be appropriate, because the peptide is endowed with several effects that are relevant to inflammation. Intracutaneous injection of PACAP causes vasodilatation (Warren et al., 1992), and in the eye, PACAP induces responses mimicking signs of inflammation, such as conjunctival hyperemia, swelling of the anterior segment in the eye, miosis, and breakdown of the blood–aqueous barrier (Wang et al., 1995).

## Islet Amyloid Polypeptide

### *Background*

Since islet amyloid polypeptide (IAPP; also known as "amylin") has previously not been implicated in neurobiology, we will briefly review what is known about this neurohormonal peptide from studies in other fields. IAPP was initially discovered as the component peptide of amyloid deposits in a human insulinoma (Westermark et al., 1986) and in pancreatic islets in type 2 diabetic patients (Cooper et al., 1987). The composition of this amyloid, which is the only morphologically apparent lesion in type 2 diabetes mellitus (Westermark, 1973) and which forms β-pleated sheet structures similar to that of β-amyloid in the plaques of Alzheimer's disease (Westermark, 1994), had eluded researchers ever since its first description almost a century ago (Opie, 1901). The extracted 37 amino acid polypeptide displayed approx 50% sequence similarity to CGRP (Westermark et al., 1986; Cooper et al., 1987), sharing an N-terminal disulfide bridge and C-terminal amidation with CGRP. Subsequent cloning revealed the existence of a single gene on chromosome 12 (Nishi et al., 1989). IAPP is expressed in pancreatic β-cells (Westermark et al., 1987; Mulder et al., 1993), localized together with insulin in the dense core granules of these cells (Johnson et al., 1988). Despite considerable efforts, it is still not clear if and how IAPP and amyloid formation contribute to the pathogenesis of type 2 diabetes mellitus; amyloid occurs in islets of both healthy and diabetic subjects (Clark et al., 1996), although being considerably more prevalent and extensive in islets from diabetic patients. It is, however, known that certain amino acid residues in IAPP 20–29 are critical for amyloid formation (Westermark et al., 1990). Interestingly, two proline substitutions in this part of rodent IAPP hinder amyloid formation (Westermark et al., 1990), which hence does not take place in rodents. Recently, overexpression of the human, amyloid-forming IAPP gene in mouse islet β-cells has revived interest in the pathogenetic role of IAPP in diabetes development; in these models, when coupled with obesity, amyloid is formed and the mice develop diabetes (Verchere et al., 1996; Soeller et al., 1998). Although it has been concluded that the rate of IAPP biosynthesis is important for amyloid formation in these animal models, its precise mechanism remains to be elucidated, but may prove to be critical for the understanding of the pathogenesis of type 2 diabetes.

Also, the biological role of IAPP has proven hard to establish (Westermark et al., 1992). A number of effects have been ascribed to the peptide; for instance, its role in the regulation of insulin secretion has been extensively studied, most studies showing that IAPP inhibits insulin secretion (cf. Ar'Rajab and Abrén 1991); such a role for IAPP has received support from analysis of the metabolic phenotype of IAPP-deficient mice (Gebre-Medhin et al., 1998a). IAPP has also been implicated in peripheral disposal of glucose; an early study showed that IAPP inhibits insulin-stimulated glycogen synthesis in rat soleus muscle strips (Leighton and Cooper, 1988), suggesting that IAPP may be a novel β-cell hormone involved in the development of insulin resistance, a hallmark of type 2 diabetes mellitus. A major obstacle to the understanding of the physiological role of IAPP is the hitherto unsuccessful identification of an IAPP-specific receptor. Although IAPP can activate CGRP receptors, conceivably owing to the structural similarities of the two peptides, it does so several-fold less efficiently than CGRP (Poyner, 1995). The CGRP receptors cloned so far are not activated by IAPP (Kapas and Clark, 1995; Aiyar et al., 1996), but there may be others. An IAPP-preferring binding site, however, has been identified in the brain (Beaumont et al., 1993; Veale et al., 1994), and the cloning of this putative IAPP receptor is eagerly awaited. In addition to the glucose homeostatic effects of IAPP reviewed here, IAPP may also be involved in calcium metabolism and satiety (reviewed in Cooper, 1994) and inhibition of gastric emptying (reviewed in Young, 1997).

### *IAPP in Primary Sensory Neurons*

Expression of IAPP is not restricted to pancreatic islets; IAPP-containing endocrine cells are encountered throughout the gut (Mulder et al., 1997a). Although it was shown early that extracts from rat DRG also contained IAPP mRNA (Ferrier et al., 1989; Nicholl et al., 1992), the localization and distribution of IAPP in sensory neurons had remained elusive. Employing a mixture of two novel oligoprobes and a modified protocol for *in situ* hybridization (adapted from Dagerlind, 1992), a novel monoclonal IAPP antibody made available to us (Percy et al., 1996) and RIA/high-performance liquid chromatography (HPLC), we were able to characterize IAPP expression in primary sensory neurons. IAPP mRNA was found in ≈25% of DRG nerve cell bodies, which mainly were of small to medium size (Fig. 5); IAPP mRNA occurred also in the trigeminal and jugular-nodose ganglia. Hybridization in adjacent sections showed that IAPP and CGRP mRNA were frequently colocalized in the same neuron. Double immunofluorescence confirmed these findings; IAPP colocalized extensively with CGRP, but was also found in SP- and PACAP-containing nerve cell bodies (Fig. 6). IAPP-immunoreactive nerve fibers were encountered in the superficial layers of the dorsal horns in the spinal cord (Fig. 7) and in a sparse supply of nerve fibers in some tissues known to receive sensory innervation, e.g., epithelium from nose and tongue or in the urinary bladder. In extracts from pooled DRGs and different levels of the spinal cord, small amounts of immunoreactive IAPP were measured (≈4 pmol/g). Reverse-phase HPLC identified two peaks of eluted immunoreactivity; the first, and smallest, peak coeluted with authentic rat IAPP, whereas the second corresponded to rat α-CGRP. This is explained by the fact that the polyclonal antibody used in the RIA crossreacted slightly with CGRP (approx 0.4%; Westermark, unpublished result). Although the exact concentration of IAPP in the DRG and spinal cord thus remains unresolved, it is conceivably very low (in the

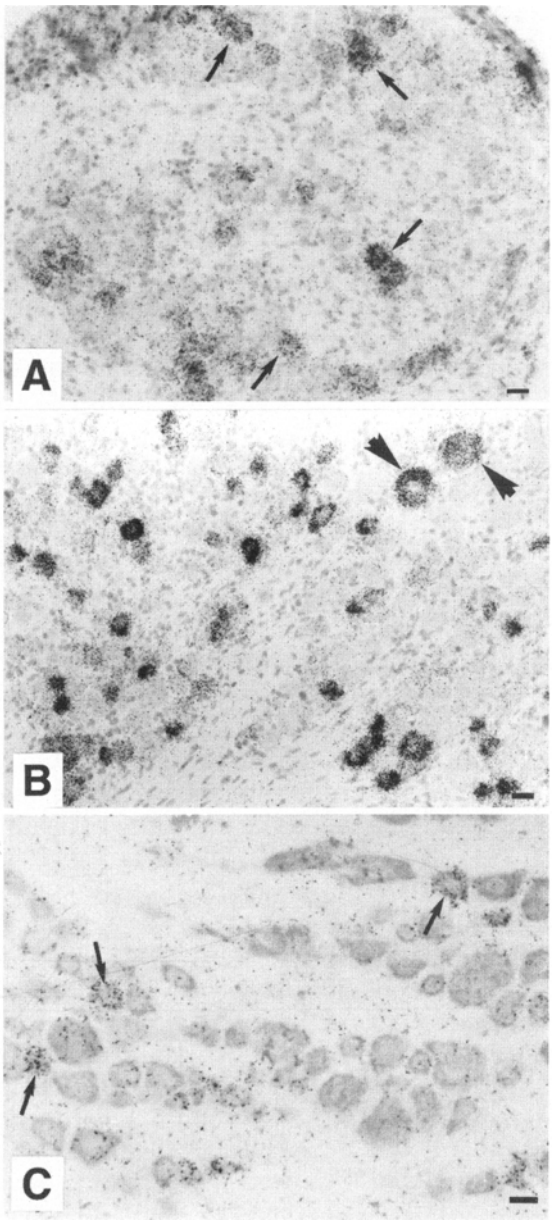


Fig. 5. *In situ* hybridization to IAPP mRNA in rat DRG **(A)** and trigeminal ganglion **(C)** and to CGRP mRNA in DRG **(B)**. IAPP mRNA is predominantly expressed in small- to medium-sized nerve cell bodies (A, C; examplified by arrows); CGRP is expressed in a greater number of nerve cell bodies, occurring also in large-sized nerve cell bodies (arrowheads). Reproduced by kind permission from the Society for Neuroscience. Bar = 20 μm.

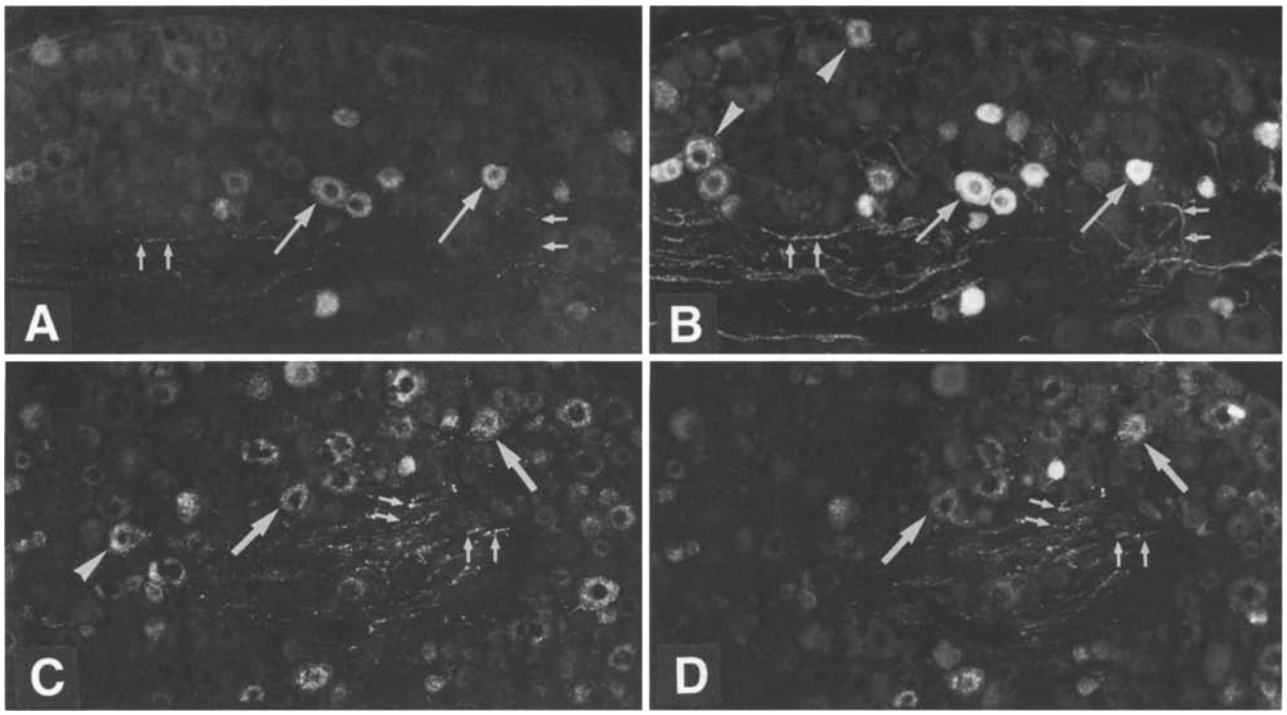


Fig. 6. Double immunofluorescence for IAPP **(A, C),** CGRP **(B),** and SP **(D)** in rat DRG (A–D). IAPP colocalizes extensively with CGRP (A, B) in nerve cell bodies (arrows) and fibers (small arrows); some CGRP-containing nerve cell bodies lack IAPP (B; arrowheads). IAPP is also colocalized with SP in nerve cell bodies and fibers (C, D; arrows); a few IAPP-containing neurons lack SP (C; arrowhead). Reproduced by kind permission from the Society for Neuroscience.

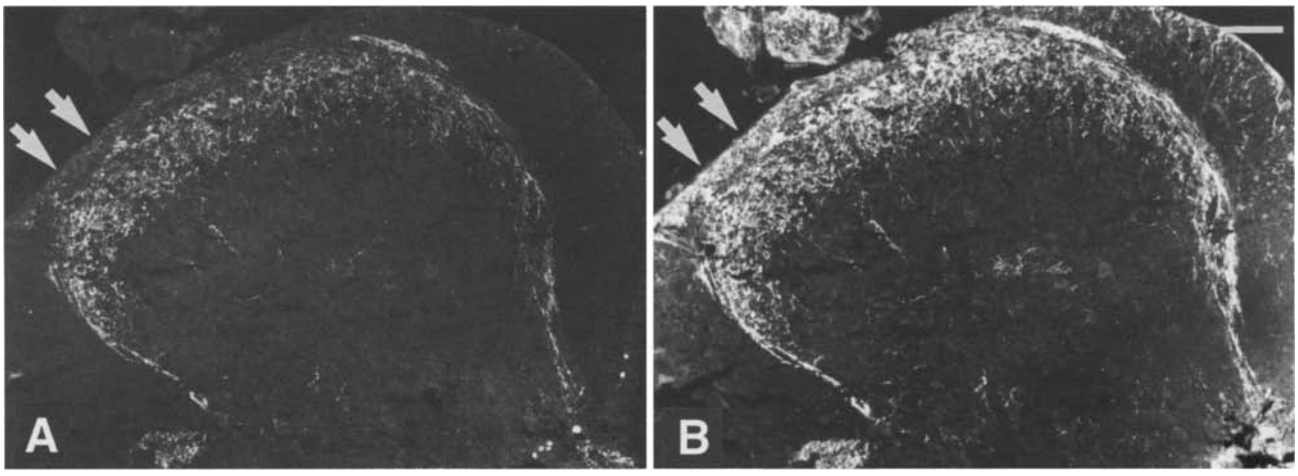


Fig. 7. Double immunofluorescence for IAPP **(A)** and CGRP **(B)** in rat spinal cord. The peptides colocalize extensively in nerve fibers terminating in the laminae I and II in the dorsal horn. Bold arrows indicate Lissauer's tract, in which IAPP is largely absent. Reproduced by kind permission from the Society for Neuroscience. Bar = 100 μm.

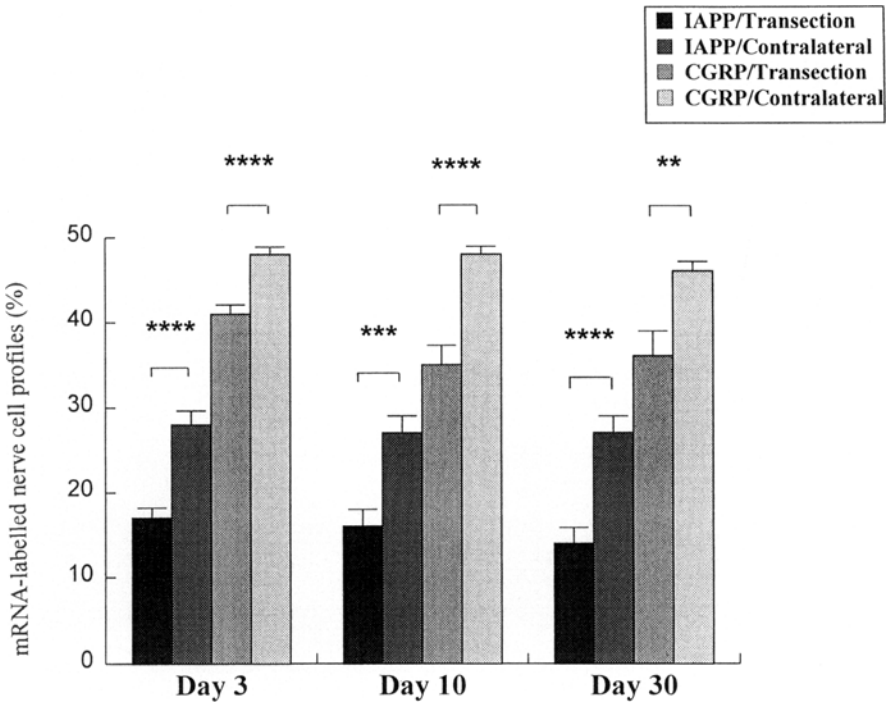


Fig. 8. IAPP and CGRP mRNA-labelled nerve cell profiles in L4–L5 DRGs on the side of sciatic nerve transection and the contralateral uninjured side. Numbers (mean ± SEM) are given as percent of the total number of nerve cell profiles in sections of L4–L5 DRGs. The percentage of nerve cell profiles on the side of sciatic nerve transection and on the contralateral uninjured side at each time-point, and for each mRNA were compared with Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data from Mulder et al. (1997b); reproduced by kind permission from Elsevier Science.

range of 1–2 pmol/g), constituting ≈1% of that previously reported for CGRP (Mulderry et al., 1988). Similarly, despite *in situ* hybridization not being quantitative in this study, it was still clear from these experiments that CGRP mRNA was expressed at a much higher level.

The distribution pattern of IAPP found in primary sensory neurons is reminiscent of that previously described for several sensory neuropeptides (cf. Sundler et al., 1985; Hökfelt et al., 1994). Moreover, the similar expression pattern of IAPP and CGRP, whose genes most likely have arisen as a result of a duplication of an ancestral gene (Nishi et al., 1989), may be a result of their retaining regulatory elements directing their tissue expression to the same cells; coexpression of IAPP and CGRP occurs also in islet δ-cells (Mulder et al., 1995a). The possibility of crossreactions, both at the level of mRNA and peptide, with CGRP was a major concern in our studies. However, the three different methods employed all supported the view that authentic IAPP expression indeed occurs in primary sensory neurons.

### *Expression of IAPP on Axotomy*

In a first attempt to find a context in which sensory IAPP plays a role, we examined the expression of IAPP and CGRP on sciatic axotomy and subsequent suture of the sectioned nerve (Mulder et al., 1997b). We found that the percentages of both IAPP and CGRP mRNA-containing nerve cell bodies were significantly reduced in the L4-L5 DRGs on the side of sciatic nerve transection at d 3, 10, and 30 follow-

ing nerve transection (Fig. 8). The findings were confirmed by immunofluorescence for IAPP and CGRP in the DRG and, as for *in situ* hybridization, occurred regardless of whether the nerve was repaired or not. In addition, a reduced density of IAPP- and CGRP-immunoreactive nerve fibers was found in the ipsilateral dorsal horn. Using quantitative *in situ* hybridization, the mean levels of IAPP and CGRP mRNA in DRG neurons were found to be significantly decreased on the injured side of those rats subjected to sciatic nerve transection alone at all time-points studied. In contrast, in rats subjected to sciatic nerve transection followed by epineurial nerve suture, the mean levels of IAPP and CGRP mRNA in DRGs were not significantly different between the transected and contralateral uninjured side.

Thus, IAPP and CGRP expression are coordinately downregulated on axotomy. For CGRP, this is in agreement with several studies (Doughty et al., 1991; Noguchi et al., 1993; Nothias et al., 1993; Zhang et al., 1993a, 1995b; Verge et al., 1995). A long-standing debate has been whether death of sensory neurons in response to nerve injury (for a review, *see* Aldskogius et al., 1985) causes the downregulation of neuropeptide expression, such as that of CGRP, or whether their phenotype is altered (*see* Hökfelt et al., 1994). This has not been resolved by our studies, but a few issues deserve to be discussed. A number of factors have been identified that affect the survival of primary sensory neurons after injury. Among these are the age of the animal and time-point at which it is studied (Himes and Tessler, 1989; Nothias et al., 1993), the proximity of the lesion to the nerve cell body and the type of nerve (Lieberman, 1971), as well as individual and species variations (Tessler et al., 1985). It is unlikely, however, that neuronal death solely will explain messenger plasticity on nerve injury. First, a marked upregulation of a number of sensory neuropeptides is seen (*see* Hökfelt et al., 1994), indicating messenger plasticity. Second, it was found in a previous study (Doughty et al., 1991), that expression of CGRP in ipsilateral DRGs at 14 d after sciatic axotomy is undetectable, which is not likely to be the result of neuronal death alone. Clearly, both neuronal death and messenger plasticity take place. The specific conditions of the model will determine to what extent one or the other contributes to the neuropeptide phenotype on axotomy. In our model, the number of nerve cell profiles (labeled and unlabeled) determined in sections of the ipsilateral DRGs at the time-points studied is not different from those in DRGs of the contralateral uninjured side (unpublished data). Taking that into consideration, it is likely that the reduction in number of IAPP and CGRP-expressing nerve cell profiles in our model predominantly is owing to phenotypic alterations of the sensory neurons. A phenotypic shift could also explain the reduced density of IAPP- and CGRP-immunoreactive fibers in the ipsilateral dorsal horn observed by us. Alternatively, although less likely, the reduced IAPP and CGRP immunoreactivity in ipsilateral dorsal horn fibers could be a result of augmented release of peptide from the terminals.

An interesting finding, although not pertaining specifically to IAPP, was that subsequent repair of the sectioned nerve maintained or restored mRNA levels of the peptides in the individual nerve cell bodies. Since this procedure facilitates nerve regeneration (Urabe et al., 1995), it could be speculated that a factor involved in such events is responsible for the maintained or restored mRNA levels. A candidate is nerve growth factor (NGF). NGF is synthesized by the part of the axon undergoing Wallerian degeneration (Heumann et al., 1987) and by nonneuronal cells in the distal stump (Korsching and Thoenen, 1983; Heumann et al., 1987). NGF increases the expression of SP and CGRP in cultured adult sensory neurons (Lindsay and Harmar, 1989), and applied to the distal sciatic nerve stump, counteracts the reduction of SP content in the spinal cord and actually increases that of the ipsilateral DRG (Fitzgerald et al., 1985). Furthermore, intrathecal NGF infusion inhibits axotomy-induced downregulation of CGRP and other neuropep-

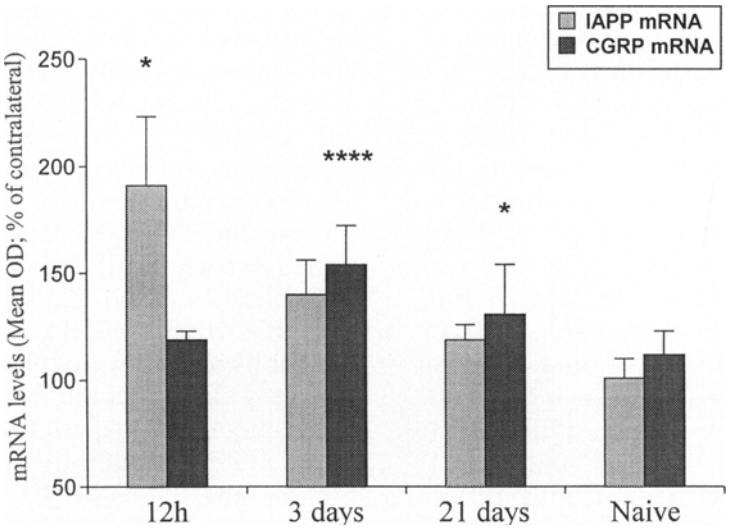


Fig. 9. The level of IAPP and CGRP mRNA (mean OD ± SEM) in L5 DRG on adjuvant-induced inflammation as determined by quantitative *in situ* hybridization; mean optical density of labeling expressed as percent of that in the contralateral control DRG; comparisons were made at each time-point with paired one-tailed Student's *t*-test. $*p < 0.05$; $****p < 0.0001$. Data from Mulder et al. (1997c); reproduced by kind permission from Elsevier Science.

tides (Verge et al., 1995). It is conceivable that suture of the nerve after axotomy will increase the availability of such a factor to the regenerating neurons and may thus affect peptide expression.

### *Expression of IAPP on Adjuvant-Induced Inflammation*

CGRP is considered to be involved in neurogenic inflammation (reviewed in Holzer, 1988); e.g., CGRP expression is upregulated in innervating sensory neurons after unilateral adjuvant-induced inflammation (Donaldson et al., 1992). Given the structural similarity of IAPP and CGRP and that IAPP can interact with CGRP receptors (for a review, *see* Poyner, 1995), we hypothesized that IAPP in sensory neurons could also play a role in neurogenic inflammation and that alterations in IAPP expression in response to inflammation may indicate that IAPP, in fact, plays such a role. To this end, we studied the expression of IAPP innervating DRG in rats in which a localized inflammation in the tarso-tibial region was induced by injection of FCA (Mulder et al., 1997c). Already at 12 h, the mean level of IAPP mRNA in the ipsilateral L5 DRG neurons was significantly increased; at d 3 and 21, the IAPP mRNA levels were still higher on the ipsilateral side, but this did not reach statistical significance (Fig. 9). CGRP displayed similar changes, although compared with those of IAPP, they were delayed and more sustained. At 12 h and d 3, also the percentages of nerve cell bodies expressing IAPP and CGRP mRNA were increased. The trend of a more sustained increase in CGRP expression is reflected by a greater percentage of CGRP-expressing nerve cell bodies on the ipsilateral side at d 21, although it did not reach statistical significance ($p = .053$). Immunocytochemistry for IAPP and CGRP confirmed the observations from *in situ* hybridization. In addition, the density of

IAPP- and CGRP-immunoreactive fibers in the ipsilateral dorsal horn was found to be increased at d 3.

Thus, as is the case for CGRP (cf. Donaldson et al., 1992; Hanesch et al., 1993), the expression of IAPP is also upregulated in response to local inflammation. However, in contrast to nerve injury (Mulder et al., 1997b), there appears to be a slightly dissociated regulation of the two mRNAs; the upregulation of IAPP is rapid but transient, whereas that of CGRP is delayed but more sustained. This differential regulation suggests that the roles of IAPP and CGRP may also be different in neurogenic inflammation. Nevertheless, such a regulation is consistent with a role for both IAPP and CGRP in inflammatory mechanisms. Again, as has been shown previously (Chin et al., 1994), these effects may be mediated by activation of a shared receptor and are likely to include vasodilatation (Brain et al., 1990), a hallmark of neurogenic inflammation (Holzer, 1988).

### *Functional Implications of IAPP in Primary Sensory Neurons—Studies in IAPP-Deficient Mice*

Although the role of IAPP in primary sensory neurons so far has not been resolved by our studies, some clues have been provided. IAPP is predominantly expressed in small-sized nerve cell bodies (Mulder et al., 1995a), thus implicating the peptide in nociception (Hökfelt et al., 1994). Moreover, most pronociceptive peptides are excitatory transmitters, as is the case for CGRP. That IAPP is also an excitatory peptide is suggested by our finding of downregulation of IAPP expression, like that of CGRP, after axotomy (Mulder et al., 1997b); it has previously been recognized that excitatory peptides as a rule are downregulated after nerve injury (Hökfelt et al., 1994). Because there is a marked structural relationship of IAPP and CGRP (Westermark et al., 1986; Cooper et al., 1987) and IAPP has been shown to act at CGRP receptors (for a review, *see* Poyner, 1995), it could be speculated that IAPP shares some of the functions of CGRP (cf. Brain et al., 1985). Indeed, IAPP is a moderately effective vasodilator (Brain et al., 1990), probably acting at CGRP1 receptors (Chin et al., 1994), and could thus be involved in neurogenic inflammation. IAPP has also been shown to augment the inflammatory activities of eosinophils (Hom et al., 1995). In contrast, however, another study showed that IAPP, like CGRP, reduces the exudate in some experimental models of inflammation and that these effects are blocked by the CGRP1 receptor antagonist $hCGRP_{8-37}$ (Clementi et al., 1995). That IAPP is involved in neurogenic inflammation is further supported by our findings of IAPP expression being upregulated in response to adjuvant-induced inflammation (Mulder et al., 1997c).

Admittedly, the evidence available for any role of IAPP in primary sensory neurons is circumstantial. However, the recent generation of IAPP-deficient mice (Gebre-Medhin et al., 1998a) allowed us to address directly whether IAPP has a role in primary sensory neurons (Gebre-Medhin et al., 1998b). As expected, immunoreactive IAPP was absent from the spinal cord and DRG in IAPP-deficient mice. Given that IAPP is predominantly expressed in small-sized nerve cell bodies (Mulder et al., 1995a), we subjected the mice to the formalin test. In this well-characterized model of nociception (Tjölsen et al., 1992), mice or rats are injected with formalin unilaterally into the dorsal hindpaw. An early and a late response are recorded by measuring the licking time of the affected paw, the late response in particular considered to reflect inflammatory mechanisms. In IAPP-deficient mice, the late phase of the formalin test was reduced, indicating that lack of IAPP from primary sensory neurons is associated with reduced nociception (Fig. 10). This suggests that IAPP is under normal conditions involved in pronociceptive transmission in primary sensory neurons and agrees with the expression of IAPP in small-sized nerve cell bodies in sensory ganglia (Mulder et al., 1995a). It is also in accord with the down-regulation of IAPP expression upon axotomy (Mulder et al., 1997b), which indicates that IAPP

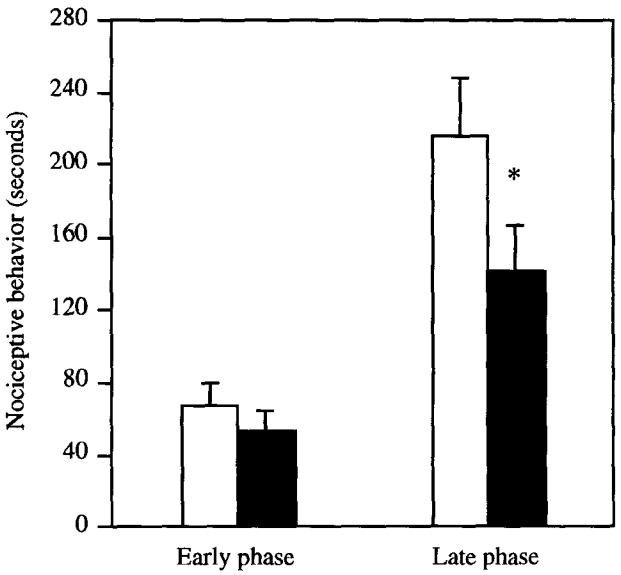


Fig. 10. Formalin test in IAPP-deficient mice. Nociceptive behavior determined at time of licking formalin-injected paw. Early phase = 0–5 min; late phase = 10–30 min. Wild-type mice—open bars; filled bars—IAPP-deficient mice. The data were compared with a two-tailed Mann-Whitney U-test; $*P = 0.024$. Data from Gebre-Medhin et al. (1998b); reproduced by kind permission from Elsevier Science.

may be an excitatory peptide messenger under normal conditions, as are the other peptides involved in nociception. Finally, the upregulation of IAPP expression in adjuvant-induced inflammation (Mulder et al., 1997c) fits with the late phase of the formalin test being reduced, since this phase mainly reflects inflammatory mechanisms.

To assess at which level the lack of IAPP mainly affected nociception in the mice, we induced localized unilateral inflammation with FCA (Larson et al., 1986). In this experiment, we were not able to discern any difference in the local impact of FCA; IAPP-deficient and wild-type mice displayed a similar degree of inflammation (Gebre-Medhin et al., 1998b), evaluated as ankle circumference, implying that the differential nociceptive response of the mice conceivably occurs at the level of the spinal cord.

## Concluding Remarks

In this article, we have reviewed the expression of PACAP and IAPP in primary sensory neurons; both peptides display a distribution characteristic of sensory neuropeptides. On nerve injury and inflammation, expression of both peptides is altered rapidly, suggesting that both peptides are involved in the response to such pathologies. Noteworthy in particular is the fact that PACAP expression is upregulated under both experimental conditions. It has previously been found that excitatory peptides are upregulated in response to inflammation and downregulated on axotomy (Hökfelt et al., 1994). PACAP, however, has been found to excite dorsal horn neurons (Dickinson et al., 1997). Thus, PACAP, perhaps not exclusively, does not fit into the established paradigm, and its potential role should perhaps be sought after in a context in which other sensory neuropeptides do not commonly occur, e.g., neuroprotection/neuroregeneration.

A major impediment to the understanding of the role of IAPP, in any context, is the lack of an identified receptor, precluding detailed studies on the mechanism for IAPP actions. That IAPP, in fact, does have a role in primary sensory neurons is corroborated by the findings of a decreased nociceptive response in IAPP-deficient mice (Gebre-Medhin et al., 1998b). Further studies in these mice may hopefully shed more light on the role of this putative sensory neuropeptide. Also, for PACAP, mice with targeted disruptions of the genes for PACAP or of its receptors (West et al., 1998) hold great promise for future studies on the physiological role of PACAP in primary sensory neurons.

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